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Evidence for Ammonia as a Natural Cue for Recruitment of Oyster Larvae to Oyster Beds in a Georgia Salt Marsh

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Abstract. Competent veliger larvae of the oysters *Crassostrea virginica* and *C. gigas* exhibited settlement behavior when exposed to ammonia (NH_3). The threshold for this response decreased with increasing larval age. The response of veligers to adult-conditioned seawater was correlated with the concentration of NH_3 in the seawater. Although the concentrations of NH_3 found in marsh water flowing over oyster beds on Sapelo Island, Georgia, were never high enough to elicit settlement behavior from oyster larvae, the concentrations found near the substrate were sufficient to induce settlement behavior in older larvae of *C. virginica*. In addition, dilution occurs during sampling in the field and may lead one to underestimate, by a factor of 1.7 to 3.5, the actual concentration of NH_3 associated with surfaces. In conclusion, NH_3 may be an important environmental cue triggering settlement behavior of larval oysters, which, along with other substrate cues, leads to cementation and metamorphosis.

Introduction

As a prelude to attachment and metamorphosis, veliger larvae of oysters exhibit a set of specific behavioral responses known as settlement behavior. This behavior is induced by a variety of chemicals, including L-3,4-dihydroxyphenylalanine (L-DOPA) which is known to activate larval endogenous dopaminergic neural pathways (Coon *et al.*, 1985; Bonar *et al.*, 1990). Recent laboratory experiments showed that ammonia (NH_3) also induces settlement behavior of oyster larvae that are competent to undergo metamorphosis (see Coon *et al.*, 1990a), but by a different mechanism, possibly involving pH-induced depolarization of nerve cells (Coon *et al.*, 1990a,b). A similar mechanism is thought to be involved in the ammonia and bacterial induction of settlement and metamorphosis of echinoid larvae (Gilmour, 1991; pers. com.). Although most organisms release ammonia as a by-product of metabolism, and the anaerobic muds characterizing oyster habitats usually contain high concentrations of ammonia, the potential importance of ammonia from these sources for settlement of oysters is not known. The presence of bacterial films on surfaces is often correlated with oyster settlement and metamorphosis, suggesting that one or more cues associated with either the bacteria or their released metabolites is important for recruitment (Galtsoff, 1964; Crisp, 1967; Weiner *et al.*, 1989; Bonar *et al.*, 1990). In addition, marine bacteria isolated and grown in laboratory cultures release soluble cues that induce settlement and metamorphosis of oyster larvae (Fitt *et al.*, 1989, 1990). A variety of analyses of supernatants from cultures of *Shewanella colwelliana*, a bacterial species known to enhance oyster recruitment, showed that their ability to induce settlement behavior of oyster larvae was correlated with the concentration of NH_3, not with that of melanin nor of any other catechol-related intermediates (Coon and Fitt, unpub.).

Oysters are gregarious, and although many attempts have been made to elucidate factors responsible for the settlement and metamorphosis of larvae around and on adults, the responsible chemical cues have not yet been conclusively identified (Cole and Knight-Jones, 1949; Knight-Jones, 1952; Crisp, 1967; Hidu, 1969; Veitch and Hidu, 1971; Keck *et al.*, 1971; Hidu *et al.*, 1978). Because oysters and oyster reefs release ammonia (Mann, 1979;

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Boucher and Boucher-Rodoni, 1985, 1988; Dame *et al.*, 1985, 1989), such release by congeners may trigger the initial 'search behavior' portion of settlement and metamorphosis (see Coon *et al.*, 1990a, for model). Oyster larvae exposed to adult extrapallial fluid exhibit "setting behavior" within 10 min of exposure, the rapid response characteristic of larval responses to NH_3, but not to catecholamines (Hidu *et al.*, 1978, Coon *et al.*, 1990b). In addition, larvae induced with adult fluids have a higher percentage attachment on shells than larvae exposed only to seawater (Hidu *et al.*, 1978). Seawater that has been conditioned by adult oysters also significantly increases setting rates on cultch (in Hidu *et al.*, 1978). Whether adult and juvenile oysters can produce enough NH_3 to induce settlement behavior, and whether larvae respond to adult-produced NH_3, has not previously been determined.

Another important but unanswered question is whether enough ammonia is present on oyster reefs to affect larval settlement behavior. The highly productive salt marshes of the east coast of the United States are characterized by organically rich mud containing partially decomposed plant material. Oyster larvae are recruited to established oyster reefs in these biologically rich and complex environments. High concentrations of ammonia have been measured from salt-marsh oyster habitats (Stevens, 1983; Boucher and Boucher-Rodoni, 1985; Dame *et al.*, 1985, 1989), but these levels are typically 5–100 times lower than those needed for induction of settlement behavior by newly competent larvae (*cf.* Coon *et al.*, 1990b). However, oyster larvae (Fitt *et al.*, 1989; Coon *et al.*, 1990a), as well as other invertebrate larvae (Knight-Jones, 1953; Bayne, 1965; Rittschoff *et al.*, 1984, 1986; Fitt and Hofmann, 1985; Crisp, 1988; Hadfield, 1977), become more sensitive to morphogens as they age. For instance, the threshold concentration of L-DOPA to which larvae of the oyster *Crassostrea gigas* will respond decreases from 10^{-5} *M* in early competency to 10^{-6} *M* three to four weeks after the onset of competence (Coon and Fitt, unpub.). In addition, during settlement, competent veligers inevitably encounter crevices and boundary layers on and near surfaces where concentrations of chemicals originating from these substrates are higher than those in the surrounding seawater. No one, to our knowledge, has attempted to look for chemical inducers of settlement of oyster larvae in micro-habitats on oyster reefs.

We therefore hypothesized that oysters and oyster reefs may produce high enough concentrations of NH_3 to trigger settlement behavior of oyster larvae. We have tested this by quantifying NH_3 levels in and around oyster reefs and comparing these with the responses of larvae of *Crassostrea virginica* and *C. gigas* to NH_3. In addition, we investigated the decline, with larval age, in the threshold concentration of NH_3 required to induce settlement behavior. We report here that the concentration of NH_3 measured in the extensive oyster bed habitats on Sapelo Island, Georgia, overlaps the response range of competent veliger larvae, suggesting that ammonia may be an inducer of settlement behavior in nature.

Materials and Methods

Laboratory experiments

Larvae of the Pacific oyster *Crassostrea gigas* (Thunberg) were obtained from the Coast Oyster Company of Quilcene, Washington, and those of the American oyster *C. virginica* from either Horn Point Environmental Laboratory, University of Maryland, St. George Oyster Company, Piney Point, Maryland, or Virginia Institute of Marine Science, Gloucester Point, Virginia. The larvae were maintained in the laboratory as detailed in Coon *et al.* (1990a).

Settlement behavior in veliger larvae of oysters includes a well-characterized series of stereotyped maneuvers (Coon *et al.*, 1990a). These include swimming with the foot extended forward, followed by crawling on the substrate in a progression of increasingly localized behaviors, including reduced crawling speed and increased frequency of turns. This behavior is initiated in competent veliger larvae upon exposure to an appropriate soluble chemical cue, and after perception of additional substrate cues may result in cementation to the substrate. Settlement behavior of competent larvae was monitored as previously described (Coon *et al.*, 1990a). Between 20 and 50 larvae were assayed in each well of 24-well tissue culture plates (Falcon #3047) in a final volume of 1.0 ml at 20°C. Typically, six replicate wells were monitored for each experimental condition. Settlement behavior of larvae actively extending their foot was monitored in each well using a dissecting microscope every 5 min, for a 1-min interval over a 30–60 min period. Responses to concentrations of NH_4Cl (pH 7.8–8.0) ranging from 300 μM to 9 m*M* were determined with 19–30-day-old competent larvae of *C. virginica*. Competent veliger larvae are defined as being able to respond to external stimuli to trigger settlement behavior and metamorphosis. This typically develops in veliger larvae between 14–21 days post-fertilization and is usually characterized by a well-developed foot and black eye-spots. All veliger larvae used in experiments possessed well-developed eye spots (Coon *et al.*, 1990a).

Some experiments were designed to monitor the settlement behavior of competent veligers in response to adult-conditioned seawater. Adult oysters (4–10 cm in length) were scrubbed 1–2 days before the experiment with a toothbrush and 10% hypochlorite solution to remove algae, invertebrates, and bacterial films. Cleaned oysters were rinsed repeatedly to remove hypochlorite, and were allowed to sit in fresh seawater for 24–48 h.

They were then placed in acid-washed glass petri dishes containing 100 ml of 0.45 μm Millipore-filtered Instant Ocean to begin the experiment. After 12–48 h, adult-conditioned seawater was removed from the petri-dish and assayed for NH_3 concentration and its ability to induce larval settlement behavior.

Field experiments

Field work was conducted in oyster beds (=reefs) in South End Creek, adjacent to the Marine Institute of the University of Georgia on the southern end of Sapelo Island, Georgia. These oyster reefs, like many on the Georgia coast, are characterized by high densities of oysters that line the creek banks and bottoms in intertidal portions of the marsh. Settlement of larvae onto these reefs occurs sporadically throughout the spring, summer, and early fall. This particular tidal creek drains a diked marsh, and contains numerous oyster reefs along its 0.75-mile course connecting it to Doboy Sound. Water samples were taken during late spring (May 1990), summer (June 1990 and 1991, August 1991), and early fall (October 1989).

The characteristics of the marsh water overlying the oyster reefs were determined from 5–10 l water samples collected hourly for 26 h during the diurnal tidal cycle 19–20 May 1990. Temperature and pH were measured simultaneously with a portable temperature-compensated pH meter immediately after collection of the water. A refractometer was used to determine salinity, and oxygen was measured with a calibrated YSI oxygen electrode within 5 min of collection. Duplicate subsamples (5 ml) were taken for ammonia determination (below). Tide height at each collection time was calculated using a marked rope weighted at one end, calibrated to the lowest and highest water level.

Water samples for determination of natural levels of ammonia associated with the oyster reefs were collected with adjustable pipettors from three general habitats on the oyster reef. First, the interface between creekwater and the oyster reef was sampled at both high and low tides from a canoe. Second, water was collected from tidepools surrounded by oyster reefs. Third, small bodies of water surrounding exposed oysters were sampled. In each habitat, water was collected with the pipettors during low tide from three sources: (1) horizontal and vertical surfaces of adult and juvenile shells, with the resulting data combined into a category called 'shell surface'; (2) crevices between oyster shells; and (3) open water near, or above, live oyster reefs. In addition, some water samples were taken from crevices and surfaces of submerged adult oysters during an incoming tide (5–10 cm below the surface). Samples, either 250 or 100 μl, were diluted with deionized distilled water before being assayed for total ammonia (NH_3-NH_4^+) as detailed below. Because the sampling procedures disturb natural nutrient gradients, replicate samples were taken from a similar habitat (*i.e.*, shell surface, crevice between shells) at the same sampling location, but not at exactly the same position. In all cases, care was taken not to disturb the water around oysters before collection.

Collecting water samples from shell surfaces and crevices inevitably involves dilution of the immediate surface water by the adjacent seawater. To estimate this dilution effect during sampling, duplicate water samples of volumes between 25 and 1000 μl were collected from the same surfaces. Ammonia was analyzed as described below and plotted against volume sampled. A theoretical boundary layer concentration was calculated by extrapolating the data back to a zero volume sample (by linear regression). A dilution factor was calculated by dividing the total NH_3-NH_4^+ concentration at the theoretical zero ml volume by the concentration sampled.

Ammonia determination

Total NH_3-NH_4^+ concentration was measured by a modification (Wilkerson and Trench, 1986) of the phenol-hypochlorite method (Liddicoat *et al.*, 1975). Absorbance of replicate samples were read on a spectrophotometer at 640 nm after a minimum 1 h incubation in the dark to allow color development. Ammonia (NH_3) concentration was calculated from standard tables relating pH, salinity, and temperature to the proportion of NH_3 from the total NH_3-NH_4^+ content (Bower and Bidwell, 1978).

Results

Larval response to ammonia

Older larvae of *C. virginica* responded to lower concentrations of NH_3 than newly competent larvae (Fig. 1). The dose-response curves showed the typical peak in maximum number of larvae exhibiting settlement behavior in less than 10 min at the highest concentrations tested (*cf.* Coon *et al.*, 1990b), extending to 20 min or longer as the concentration of NH_3 decreased (Fig. 1). The lowest concentration of NH_3 eliciting larval settlement behavior (8.2 ± 3.3%, mean ± S.D., of larvae responding, n = 6) that was higher than controls (0%, n = 6) was 7.1 μ*M* (Fig. 2).

Larval response to adult-conditioned water

Competent larvae of both *C. gigas* and *C. virginica* exhibited settlement behavior when exposed to adult-conditioned seawater (Figs. 2, 3). The level of larval response was similar to that expected from the amount of NH_3 found in the adult-conditioned seawater (Fig. 2). In addition, the larval response to adult-conditioned water increased in a predictable fashion when the NH_3 concentration in oyster-conditioned water was increased by rais-

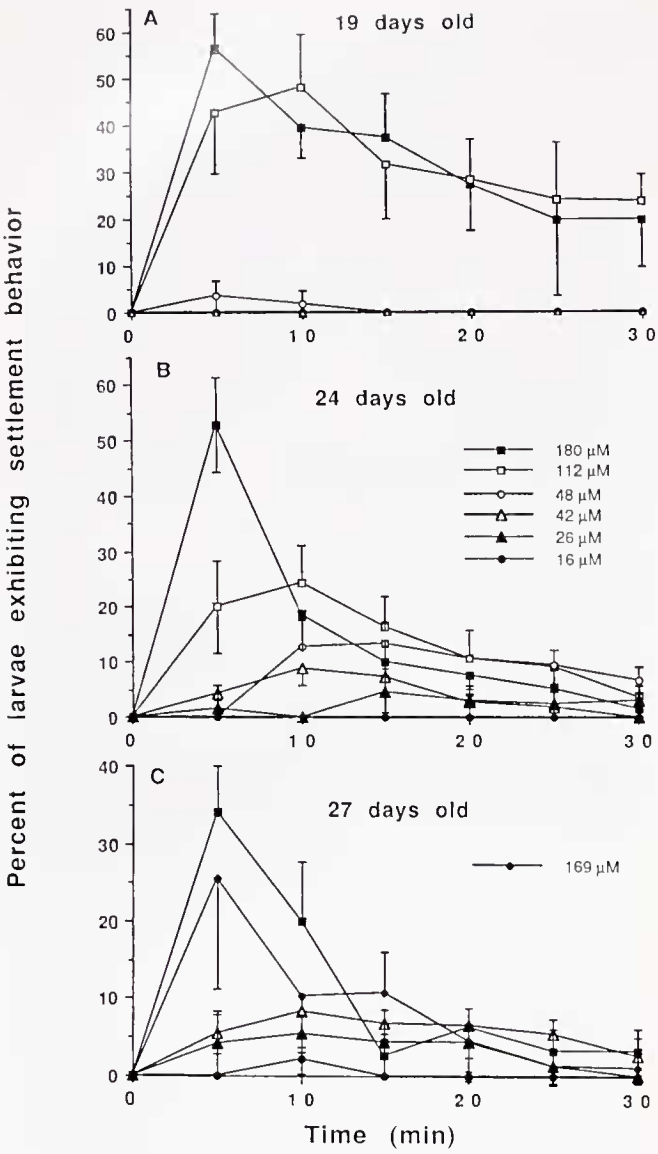

Figure 1. Percent of *Crassostrea virginica* exhibiting settlement behavior when exposed to various concentrations of NH_4Cl as a function of exposure time. All experiments were conducted at pH 7.8–8.0. Concentrations of NH_3 were approximately: solid squares = 180 μM NH_3; diamonds = 169 μM NH_3; open squares = 112 μM NH_3; open circles = 48 μM NH_3; open triangles = 42 μM NH_3; closed triangles = 26 μM NH_3; closed circles = 16 μM NH_3. Data are means ± S.D., n = 6.

ing the pH of the conditioned water from 7.4 to 8.0, and decreased by lowering the pH from 7.4 to 7.1 (Fig. 3).

Ammonia levels in an oyster bed

Total NH_3-NH_4^+ concentration in surface (0–20 cm) creek water over an oyster bed on Sapelo Island in May 1990, varied with tide height and time of day (Fig. 4). Total NH_3-NH_4^+ levels were inversely correlated with tide height, pH, and oxygen (Fig. 4). Highest concentrations of total NH_3-NH_4^+ were measured during low tide, when oxygen levels and pH were lowest. The total NH_3-NH_4^+ concentrations in these samples did not exceed 20 μM, and ammonia (NH_3) was less than 1 μM. The highest NH_3 concentrations in creek water were on an incoming tide during the daytime (Fig. 4B). There were two low points in NH_3 concentration: (1) at peak high tide, when total NH_3-NH_4^+ was at its lowest in the seawater entering the creek from Doboy Sound; and (2) on the outgoing tide, when pH decreased relatively faster than the total NH_3-NH_4^+ concentration increased (Fig. 4B).

When water samples were taken next to oyster shells, in moving water on an incoming tide, NH_3 concentrations were similar to that of the creek water overlying the oyster bed (Table I: 1, 2A3). However, water sampled from surfaces and between shells at low tide when the flow was minimal often had higher levels of NH_3 and total NH_3-NH_4^+ than the overlying creek water (Table I:1). Values

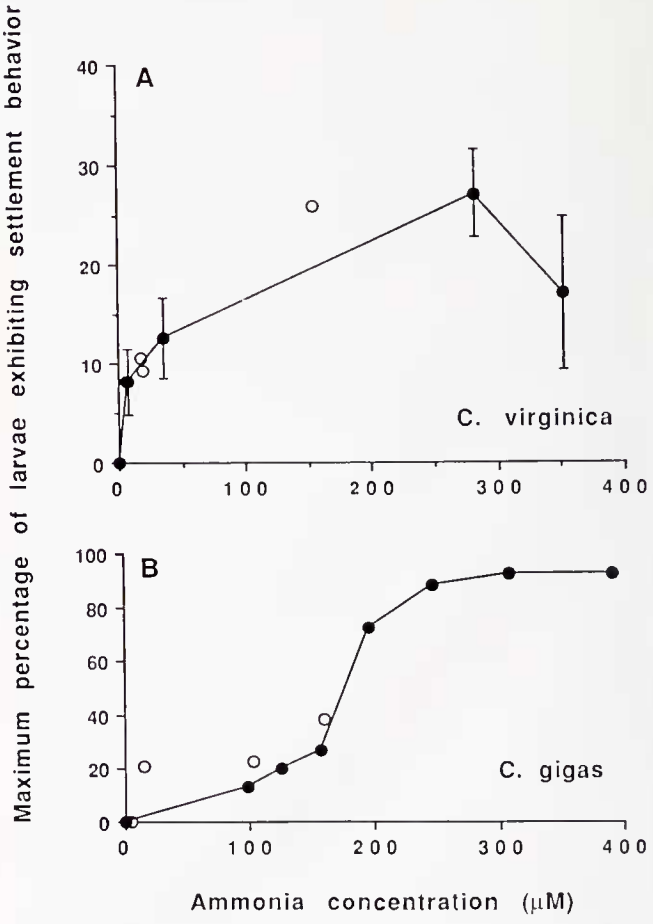

Figure 2. Maximum percent of larvae exhibiting settlement behavior in response to either NH_4Cl (filled circles) or adult-conditioned water (open circles). (A) *Crassostrea virginica*, (30 days old), (B) *C. gigas*, newly competent larvae (19 days old). Data are means ± S.D. (n = 6) of the maximum response, seen between 0 and 20 min, depending on the concentration of NH_4Cl.

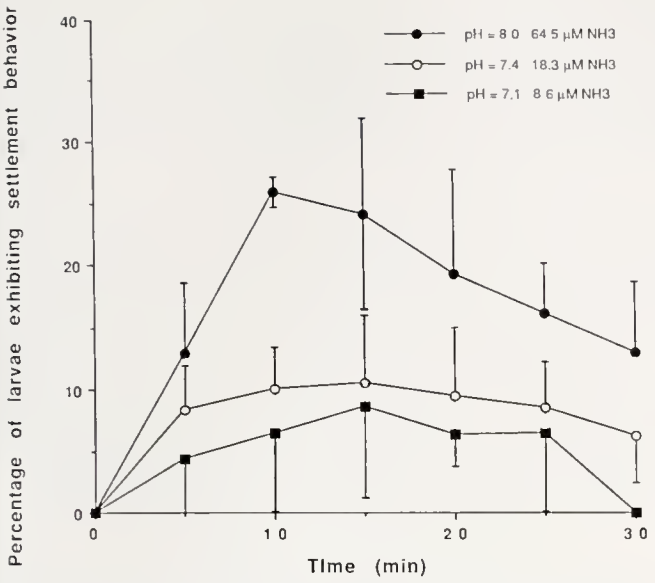

Figure 3. Percent of *Crassostrea virginica* exhibiting settlement behavior in response to adult-conditioned seawater as a function of exposure time. pH was adjusted before the experiment began in order to manipulate the NH_3 concentration, as noted in the text. Data are means ± S.D. (n = 6).

in Table 1:1 are from August 1991; values from June 1991 were similar to those found in August 1991. Samples taken at earlier dates were lower, probably due to dilution from surrounding water resulting from the larger volumes sampled (>1.0 ml), or due to seasonal differences. The highest concentrations of total NH_3-NH_4^+ were recorded between oyster shells (crevices), and include numerous samples exceeding 300 μM and a maximum concentration of 422 μM total NH_3-NH_4^+. The highest average ammonia (NH_3) values were recorded on 2 August 1991 around oysters exposed on an incoming tide (mean = 6.6 ± 3.2 S.D. μM NH_3, range 3.9 to 11.2 μM, n = 9). Six out of 16 samples taken from shell crevices on this date had concentrations of NH_3 greater than 7.1 μM. Although the total NH_3-NH_4^+ concentrations were higher on some samples on the previous afternoon, NH_3 concentration was always lower than 7.1 μM, because the pH of the outgoing tide was so low.

Dilution factor

As smaller volumes were sampled from a surface of an oyster shell, the measured total NH_3-NH_4^+ concentration increased (Fig. 5). The calculated dilution factor varied with sample volume, and was about 1.5 for volumes ≥ 250 μl using a 1000 μl pipettor (Fig. 5), and 3.5 for 100 μl samples using a 100 μl pipettor (data not shown). These dilution factors will obviously vary with type of habitat and substrate sampled. Control samples from >1 cm away from a shell surface showed no significant difference in ammonia concentration with sample volume. The highest concentrations of NH_3 measured in the oyster beds (Table 1) are well within the minimum range needed for induction of settlement behavior of older larvae of *C. virginica* (Fig. 2, above). Environmental concentrations of NH_3 associated with some shell surfaces, calculated using these dilution factors, exceeded 30 μM, far surpassing the minimum values needed to elicit larval settlement behavior.

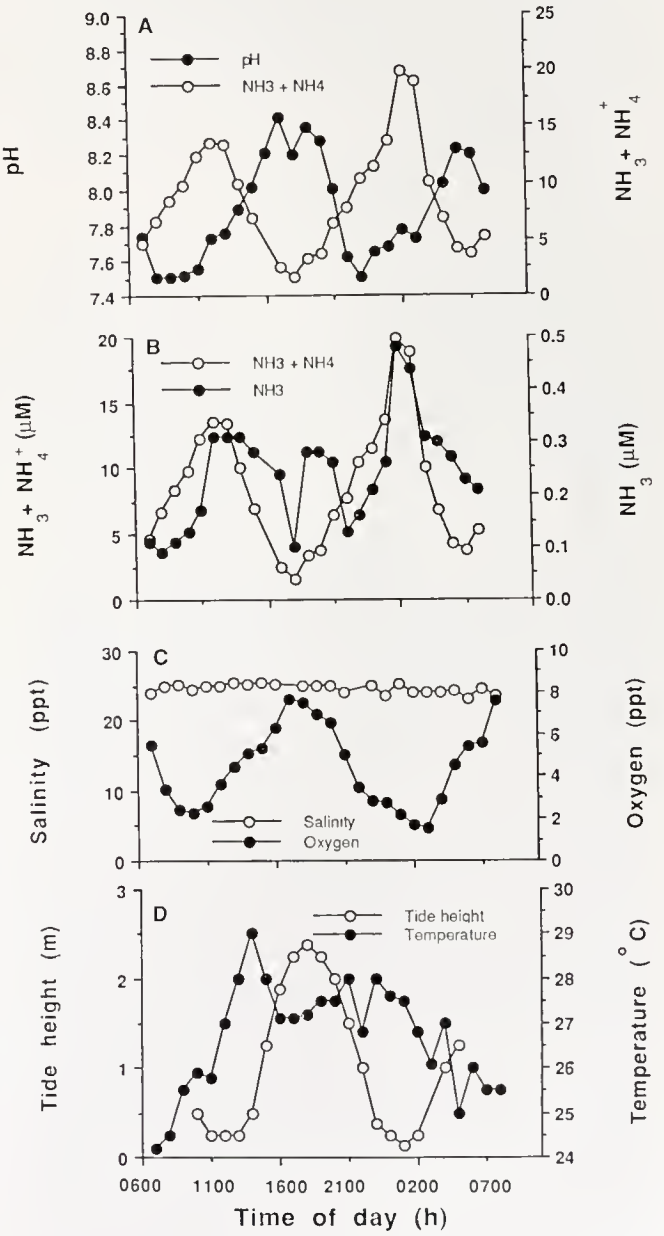

Figure 4. Characteristics of creek water from Sapelo Island on 19–20 May 1990 in relation to time of day. (A) Total NH_3-NH_4^+ and pH. (B) NH_3 and total NH_3-NH_4^+. (C) Salinity and oxygen. (D) Tide height and temperature.

Table I

Concentration of total NH_3-NH_4^+ [μM, means ± S.D. (n)], and the range of corersponding concentrations of NH_3 (μM) in oyster bed habitats on Sapelo Island

	Ambient seawater	Shell surface	Shell crevice
I. 1 August 1991 (250 µl samples)			
A. Morning low tide (incoming tide), pH = 7.4–7.7			
1. Creek	43.2 ± 2.6 (7)	52.1 ± 5.3 (14)	70.5 ± 32.5 (11)
Range (NH_3):	0.8–0.9	0.9–1.2	0.9–3.2
2. Pool 1	101.0 ± 1.7 (3)	111.3 ± 12.9 (3)	165.4 ± 66.3 (8)
Range (NH_3)	2.0–2.0	2.0–2.5	2.0–5.4
B. Afternoon low tide (outgoing tide), pH = 7.3–7.4			
1. Pool 1	81.9 ± 7.8 (4)	76.5 ± 3.5 (2)	142.6 ± 56.8 (9)
Range (NH_3):	0.7–0.9	0.7–0.8	0.9–2.6
2. Pool 2	199.4 ± 14.0 (2)	203.0 ± 11.3 (2)	270.7 ± 82.7 (6)
Range (NH_3):	1.9–2.1	2.0–2.1	2.1–4.2
3. Exposed oysters	n.d.	n.d.	256.6 ± 55.5 (5)
Range (NH_3):	n.d.	n.d.	1.7–3.3
II 2 August 1991 (100 µl samples)			
A. Morning low tide (incoming tide), pH = 7.8–8.0			
1. Pool 1	72.8 (1)	n.d.	101.3 ± 38.4 (7)
Range (NH_3):	2.8	n.d.	3.2–7.2*
2. Exposed oysters	n.d.	n.d.	169.9 ± 83.0 (9)
Range (NH_3):	n.d.	n.d.	3.9–11.2*
3. Between tides (reef underwater)	20.9 ± 6.7 (5)	18.2 ± 1.1 (2)	21.7 ± 2.1 (9)
Range (NH_3):	0.5–1.1	0.7–0.7	0.7–1.0

* Six out of 16 water samples taken from these habitats had a high enough concentration of NH_3 (≥7.1 μM) to induce settlement behavior of veliger larvae.

n.d. = no data.

Discussion

Our goal in this study was to determine whether NH_3 levels in nature are high enough to induce settlement behavior of veliger larvae of oysters. The data show that concentrations of NH_3 close to oyster shells in oyster beds at Sapelo Island reach concentrations at least as high as the minimum concentration of NH_3 needed to induce settlement behavior of larvae of *C. virginica*. In addition, adult oysters produced enough NH_3 in laboratory experiments to induce settlement behavior. These results suggest that NH_3 concentrations in or near boundary layers of surfaces in oyster beds may be at least partially responsible for triggering settlement behavior in nature.

The highest values for total NH_3-NH_4^+ were found during afternoon low tides in the summer, when temperatures are typically highest in the marsh (Table I). However, the interaction of pH of the ambient seawater and total NH_3-NH_4^+ (Fig. 4, Table I) combined to give concentrations of NH_3 that were higher on incoming than outgoing tides. If competent oyster larvae are present in the water column, and if NH_3 is one of the cues to which they respond in nature as suggested in this study, then one might expect oyster larvae to settle during incoming tides rather than on outgoing tides. An alternative scenario might find competent larvae in areas of quiescent water on oyster reefs during low tide, where levels of NH_3 may become very high. There are currently no convincing data indicating that part of the tidal cycle, or time of day, when oyster larvae tend to set.

Other chemical cues also trigger settlement behavior of oyster larvae. A number of catecholamines, including L-DOPA and norepinephrine, induce classic veliger settlement behavior and subsequent metamorphosis (Coon *et al.*, 1985, 1990a). Treatment with methylamine and other weak bases also induces settlement behavior (Coon *et al.*, 1990b). None of these compounds has been found in oyster beds, but there is evidence from experiments in salt marshes that other soluble cues may exist and play a role in oyster settlement. Zimmer-Faust (1990) and Tamburri (1990) found differences in larval swimming behavior in marsh water containing sub-threshold concentrations of NH_3. The relationship between these changes in swimming behavior and the specific behaviors involved in settlement (*e.g.*, foot extension, crawling, and turning) are unclear. While these other soluble cues may be important in oyster recruitment, their identity and characteristics are virtually unstudied.

Chemical induction of settlement behavior modifies veliger movement in such a way as to bring competent larvae into physical contact with potential substrates for

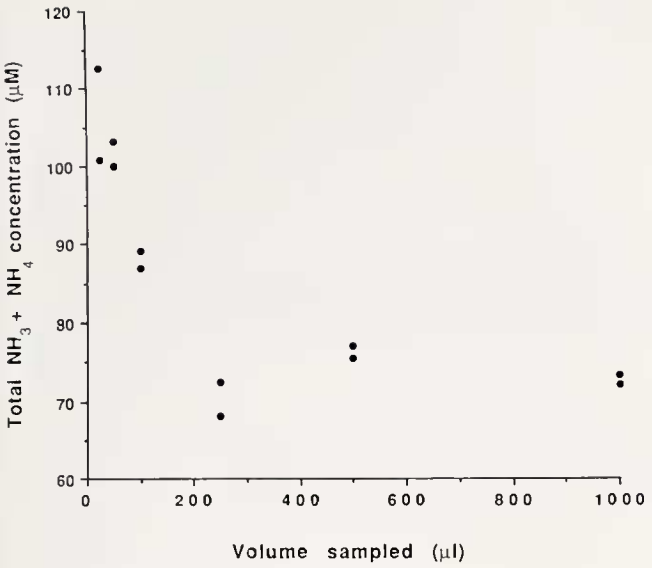

Figure 5. Relation of total NH_3-NH_4^+ measured to volume of water sampled from oyster-shell surfaces on Sapelo Island using a 1000 μl pipettor.

attachment and metamorphosis. Researchers have speculated for years about the characteristics of substrates suitable for oyster attachment and metamorphosis, but only the presence of other larvae or adults (Cole and Knight-Jones, 1949; Knight Jones, 1952; Crisp, 1974) and biofilms (Crisp and Ryland, 1960; Galtsoff, 1964; Weiner *et al.*, 1989) have convincingly correlated with higher oyster set. The molecular factors associated with conspecifics in nature are not known, but as shown here may involve the production of ammonia. Our data show that larval behavior can be altered by changing the availability of NH_3 in adult-conditioned water by changing pH. Others have found a settlement-behavior inductive factor in adult-conditioned water, but have been unable to identify it (references in Hidu *et al.*, 1978).

Settlement of oyster larvae involves two basic steps. (1) Settlement behavior triggered by soluble cues that act to bring the larvae in contact with surfaces, and (2) cementation and subsequent metamorphosis triggered by unknown cues associated with surfaces (Coon *et al.*, 1990a). The latter appear to be related to biofilms, but few experiments have addressed this relationship (*cf.* Walch *et al.*, 1987; Labare and Weiner, 1990). Many of the early results showing more set on cultch coated with oyster extracts than control cultch (references in introduction) may have been due to higher numbers of resulting bacteria, and thus higher concentrations of NH_3 as well.

Ammonia-induced settlement behavior does not by itself result in subsequent attachment and metamorphosis in laboratory experiments (Coon *et al.*, 1990b; unpub.). Experiments demonstrating this were performed in plastic cell-culture plates, previously shown to be sub-optimal setting surfaces for oyster larvae (Coon *et al.*, 1990a). Because larvae that are induced with NH_3 characteristically habituate to that stimulus in less than 30 min and then resume normal swimming behavior (Coon *et al.*, 1990b), we hypothesize that, in the laboratory, they do not spend enough time in contact with this substrate to induce settlement. Such a phenomenon may also occur in competent larvae in nature, where the importance of selecting among a variety of settlement sites may be crucial to survival. Such a process may be part of the mechanism by which veliger larvae settle on premium substrates, such as congener shells, more frequently than suboptimal substrates, such as mud. The substrate factors important in triggering final attachment and metamorphosis are not currently known.

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